POSSIBLE ROLE OF FREE RADICAL FORMATION IN CLOZAPINE INDUCED AGRANULOCYTOSIS.

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Clozapine is a unique anti-psychotic drug due to its lack of extrapyramidal side-effects and effectiveness with otherwise "treatment resistant" patients. Cases of treatment induced agranulocytosis have been reported, yet the exact mechanism is unknown. Peroxidases in neutrophils and stem cells could activate Clozapine to reactive intermediates.

Clozapine oxidation by horseradish peroxidase/hydrogen peroxide was monitored by the disappearance of the UV absorbance at 290 nm. HPLC analysis revealed the formation of at least four radioactive peaks as a result of clozapine metabolism by human myeloperoxidase. In the presence of glutathione (GSH), overall metabolism was reduced and the latter metabolite peaks were replaced by two GSH-conjugates as identified by NMR and MS-spectroscopy.

Evidence for one-electron transfer reactions i.e. the initial formation of a clozapine radical stems from spin trapping of a glutathionyl radical with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), which results in a four-line ESR signal. In addition, a DMPO-superoxide adduct was detected by ESR when GSH was replaced by NADPH and in the presence of either GSH or NADPH oxygen consumption could be monitored with a Clarke oxygen electrode.

These data suggest clozapine activation to free radicals which are able to cause oxidative stress or lead to adduct formation. Thus free radical metabolites could be related to the agranulocytosis observed in some patients.

BIOOXIDATION OF GOLD(I) TO GOLD(III) DETECTED BY T MEMORY CELLS

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Neither the pharmacological mechanism nor the mechanism underlying the frequent adverse immunological effects of gold drugs are known. All gold drugs, such as gold sodium thiomalate (GST), contain gold in the gold(I) oxidation state. Our results suggest that in mice the adverse side-effects of GST are caused by gold(III) which is generated in vivo through oxidation of gold(I). Upon weekly i.m. injections of GST, but not of ST alone we were able to reproduce several of the adverse side-effects of gold in the mouse. Although both the type of these immunopathological alterations and their association with the MHC strongly suggested that they were due to the activation by GST of T cells, no evidence for this was detectable. Interestingly, however, the mice treated with GST, i.e., gold(I), showed strong anamnestic T cell responses to gold(III). This finding suggests that the adverse immunological side-effects of gold(I) are, in fact, due to T cell reactions elicited by gold(III). Gold(III) might be generated by oxidation of gold(I) in the phago-lysosomes of phagocytic cells, possibly involving myeloperoxidase. Thus, T memory cells were used as a tracer to detect gold(III), a metabolite generated in vivo that is not or only with difficulty detectable by physico-chemical methods. Generally speaking, metabolites detected by this novel approach are likely to be relevant with respect to adverse immune reactions to drugs.

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CADMIUM ACCUMULATION AFTER DIETARY ADMINISTRATION OF $CdCl_2$ OR Cd-METALLOTHIONEIN IN RATS AND THE EFFECTS OF MINERAL SUPPLEMENTS.

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Outside of the industrial environment, most of the body burden of Cadmium (Cd) is derived from the diet. In food of animal origin, Cd is mainly bound to the protein metallothionein (Mt). There is some indication that exogenous metallothionein as present in food can survive gastro-intestinal digestion and can cross the intestinal barrier intact. Moreover, previous studies from our laboratories using CdMt incorporated in the diet have shown a clear difference in toxicity between $CdCl_2$ or CdMt. We have focused our studies on the difference in Cd disposition in rats between dietary administration of $CdCl_2$ and CdMt, and the effect of nutritional factors such as minerals. The source of CdMt was liver tissue from pigs treated with $CdCl_2$. The feeding of CdMt (3-30 mg Cd/Kg) consistently resulted in about 50 % lower Cd accumulation in liver and intestinal mucosa than the feeding of $CdCl_2$. However, both 30 mg/kg Cd-fed groups showed a similar Cd-accumulation in the kidneys until day 28. Therefore in contrast to $CdCl_2$ exposure after exogenous CdMt exposure Cd preferentially accumulates in the kidneys and not in the liver. In both liver and kidneys, Mt levels increased during the course of the study: while in liver after $CdCl_2$ intake Mt levels were higher than after CdMt intake the renal Mt concentrations were very much the same for both 30 mg/kg Cd-fed groups. In fact on day 7 CdMt administration resulted in slightly higher renal Mt levels than $CdCl_2$ administration, suggesting the direct accumulation of exogenous CdMt in the kidneys.
The uptake of Cd from $CdCl_2$ or CdMt was significantly decreased by dietary mineral supplements. The protective effect against Cd accumulation from $CdCl_2$ was mainly due to the presence of Fe (2+). However, the most pronounced effect was obtained by a combined mineral supplement of Ca/P, Zn and Fe which resulted in an 80 % reduction of the Cd accumulation in liver and kidneys. The results indicate that Cd-speciation and the mineral status of the diet have a considerable impact on the Cd accumulation in rats.

STEREOCHEMICAL ASPECTS OF THE DISPOSITION OF INDOBUFEN IN RATS, MICE AND HUMAN SUBJECTS

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Indobufen (IB; 2-[p-(1-oxo-2-isoindolinyl)phenyl]butyric acid) is a platelet aggregation inhibitor. Although IB is chiral and is administered as the racemate only the S-enantiomer is pharmacologically active. Accordingly, we have investigated the disposition of *rac*-IB and its enantiomers given orally to rats, mice and human volunteers. [^{14}C]-S-IB, [^{14}C]-*rac*-IB or racemic indobufen were dosed to rats, bile duct-cannulated rats (10 and 20 mg/ kg; S- & *rac*-IB respectively) and mice (12.5 & 25 mg/ kg) and racemic indobufen (200 mg) or S-IB (100 mg) to human volunteers. ^{14}C elimination was determined by scintillation counting. IB metabolites were separated by HPLC and quantitated by scintillation counting or relative peak area. The enantiomeric composition of IB and its 5-hydroxylated metabolite was determined after conjugate hydrolysis by HPLC and derivatization with L-leucinamide, and of the diastereoisomeric IB glucuronides by chiral HPLC. Over 90% of dosed ^{14}C was recovered from rats and mice. There was considerably higher faecal excretion of ^{14}C in rats and mice after dosing [^{14}C]-*rac*-IB (59 & 42% of dose) compared with [^{14}C]-S-IB (43 & 26%). The major route of elimination in bile-duct-cannulated rats was the bile. The IB:5-OH-IB ratio (free & conjugated, urine & faeces) after dosing rats with S-IB was 1:7.3 and *rac*-IB, 1:1.2. The metabolic pathways of *rac*- and S-IB were essentially the same in both species, but there were marked species differences between rats and mice: the major metabolites in the rat were 5-OH-IB and its sulfate (urine & bile), IB, 5-OH-IB (faeces) and IB glucuronide (bile), and in the mouse, IB glucuronide. The urinary excretion of S-IB (mice) and 5-OH-S-IB (rats) was higher than that of their R-antipodes. The elimination of the S,D-IB glucuronide was favoured in mouse urine, whereas the R,D-IB glucuronide was favoured in man.

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BIOACTIVATION OF *vic*-DIHALOALKANES BY CONJUGATION WITH GLUTATHIONE

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The pesticide and carcinogen ethylene dibromide (EDB) is activated to a mutagen by enzymatic conjugation with glutathione (GSH). Such activation is an exception to the general mode of detoxication via GSH S-transferase action. The primary DNA adduct (≥ 95%) is *S*-[2-(N^7-guanyl)ethyl]GSH and a minor adduct is *S*-[2-(N^1-adenyl)ethyl]GSH. The guanyl adduct slowly depurinates and is processed to form the mercapturic acid *N*-acetyl-*S*-[2-(N^7-guanyl)ethyl]cysteine, which is excreted in the urine and may serve as a biomarker of damage. In rat liver the level of the major DNA adduct is decreased by GSH depletion and elevated by either induction of GSH *S*-transferases (esp. 2 and 3) or inhibition of the cytochrome P-450 oxidation of EDB by disulfiram, a treatment which acerbates hepatocarcinogenicity. The unstable conjugate (half-mustard) GS-CH_2CH_2Cl has been synthesized and found to be a potent mutagen, producing bacterial base pair mutations as does EDB. Alteration of the GSH moiety has been shown to have dramatic influence on the ratio of mutants/bacterial N^7-guanyl DNA adducts. In a forward system (phage M13 *lacZ*), the half-mustard-dependent mutations were strongly dominated by GC/AT transitions. Oligonucleotides (6- and 7-mers) containing a single *S*-[2-(N^7-guanyl)ethyl]GSH moiety have been constructed: UV, CD, and NMR studies indicate that the adduct is strongly destabilizing with regard to pairing even several bases distant, but the apparent mispairing of the adduct with thymidine may be a kinetic process as opposed to thermodynamic. The principal N^7-guanyl DNA adducts derived from 1,2-dibromo-3-chloropropane have also been identified and can, as in the case of EDB, be explained in terms of S_N2 and episulfonium ion chemistry. However, the *in vivo* levels of these adducts are considerably lower than found for EDB. (Supported in part by USPHS grants CA 44353 and ES 00267)

THE TISSUE LOCALISATION OF GLUTATHIONE S-TRANSFERASES IN MAN

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The glutathione S-transferases (GSTs) are a supergene family of enzymes with a wide range of functions involving conjugation of electrophilic compounds with reduced glutathione. In addition, GST may bind nonsubstrate ligands such as bilirubin and they have been implicated in the synthesis of cysteinyl leukotrienes and steroidogenesis. In humans there are at least three distinct gene families giving rise to cytosolic isozymes and one giving rise to membrane associated, microsomal GST. We have studied the distribution of alpha, pi and mu class cytosolic GST and microsomal GST in a wide range of healthy and diseased human tissues by immunohistochemistry.

In many tissues secretory or duct type epithelium expresses GST pi. By contrast, parenchymal cells often express GST alpha but not GST pi. In alcoholic cirrhosis hepatocytes express GST pi in addition to GST alpha - this appears to be relatively specific for alcohol since other causes of cirrhosis do not induce this change. In glomerulonephritis renal tubules lose expression of GST alpha and reexpress GST pi, which is normally present in fetal kidney. GST mu is known to be polymorphic in man. Microsomal GST may also be polymorphic.

GST pi has been implicated in tumourigenesis in animals although models of tumour progression such as liver cancer and cervical carcinoma do not confirm this in man. GST has been implicated in tumour cell drug resistance. Small cell carcinoma of lung and Wilms' tumour express little GST and are chemosensitive whereas lung adenocarcinoma and renal cell carcinoma, which are chemoresistant, express much GST, but a direct role for GST remains unproved.

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MECHANISM-BASED HAZARD ASSESSMENT

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Hazard assessment is a first-step prerequisite to risk assessment and evaluation. If no human data are available as with any newly developed chemical, hazard assessment has to be based on animal data and in vitro findings. Interspecies differences in susceptibility and sensitivity mostly do not allow for direct extrapolation from animals to humans. Elucidation of the mechanism of action on the cellular and molecular level may fill the gap. Toxicokinetics and metabolic activation and deactivation reactions play a dominant role. A variety of examples how this may be done have been published, one of which will be dealt with in detail: nephrotoxicity and -carcinogenicity of haloolefins (e.g. tri-, tetrachloroethene, dichloroacetylene, hexachlorobutadiene). Bioactivation starts with glutathione adduction, followed by the formation of the respective cysteine adducts which are cleaved by a specific enzyme, ß-lyase, which is highly concentrated in the target renal tubular epithelia. The ultimate electrophilic intermediates have been identified as halothioketenes, or thioacyl halides which interact covalently with DNA bases, proteins and other macromolecules by acylation. An interspecies comparison of the reaction rates of the single bioactivation steps allows for a prediction of toxicity and carcinogenicity in humans exposed to these compounds.

BIOTRANSFORMATION IN INVERTEBRATES AND PLANTS

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The biotransformation pathways of xenobiotics in invertebrates and plants are similar to those in mammals with certain exceptions which will be emphasised. An important distinction between plants and mammals is that the former have no effective excretory mechanism. Plant metabolites of herbicides, insecticides, etc., therefore, are stored within plant tissue as terminal metabolites along with the natural array of secondary metabolites. Much effort is expended on the identification, measurement and toxicological assessment of terminal crop metabolites during the development of a plant protection agent. One of the most important aspects of biotransformation in plants is its role in selective toxicity. Much of the selectivity in the action of herbicides between crop plants and competing weeds is due to differences in rates or routes of biotransformation. Such a difference may be in the inactivation of a herbicide or in the bioactivation of a pro-herbicide. Similarly, selectivity of action between insect pests and beneficials can often be explained thus. Where insecticides are designed to act on the nervous system, selectivity between insect and mammal is also an important consideration. In the context of environmental contamination by chemicals generally, plants and insects per se destroy an insignificant proportion of the total chemical challenge. In fact, plants create considerably more chemicals than they destroy. Plants, however, are vital, via their maintenance of the biologically-active rhizosphere and soil quality, in the quantitatively most important detoxification process - that which is effected by the various microorganisms in soil. These various aspects will be exemplified.

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DISCOVERY, EVALUATION AND DISPOSITION OF NOVEL PLATINUM DRUGS

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The widespread registration of carboplatin (Paraplatin) has made available an alternative to cisplatin which is essentially devoid of the major side effects of the latter, notably nephrotoxicity, ototoxicity and neuropathy. Carboplatin is less emetogenic than cisplatin and its dose-limiting toxicity is myelosuppression, particularly thrombocytopenia. In ovarian cancer, response rates (approx. 50%), relapse rates and disease-free intervals are broadly comparable for both drugs. Thus carboplatin has made no impact on the prevalence of either intrinsic or acquired resistance to platinum therapy in ovarian cancer. In approaching this problem we have developed panels of human ovarian carcinoma cell lines and their xenograft counterparts. Cytotoxicity differences of almost two orders of magnitude are apparent between the most sensitive and most refractory (intrinsically resistant) lines. Acquired resistant variants of the most sensitive lines have also been developed. A new class of PtIV coordination complexes, platinum IV ammine/amine dicarboxylates has been developed, general formula $[PtCl_2(OCOR_1)_2NH_3(RNH_2)]$, where R_1 = aliphatic or aromatic, R = aliphatic or alicyclic. These compounds are generally well absorbed from the GI tract, unlike cisplatin and carboplatin. For example, in an homologous series where R = cyclohexyl and R_1 = CH_3-C_3H_7, oral (mouse) bioavailabilities were: JM216 (CH_3), 45%; JM231 (C_2H_5), 78%; JM221 (C_3H_7), 91%. In a panel of human ovarian carcinoma cell lines, some complexes were selectively more cytotoxic to lines highly resistant to cisplatin (CDDP) (eg $IC_{50}\mu M$: line HX/62 — CDDP 13.3, JM221 0.19 (70-fold enhancement); line SKOV3 — CDDP 5.8, JM221 0.10 (58-fold enhancement), whereas line 41M — CDDP 0.20, JM221 0.05 (only 4-fold enhancement)). Many of these compounds exhibit antitumour activity (po) comparable to or greater than that of cisplatin (iv): eg. in the ADJ/PC6 plasmacytoma TI CDDP = 18, TI JM221 = 54; in human ovarian carcinoma xenografts, line PXN/100 — both cisplatin and JM221 are curative; line OVCAR-3, T/C CDDP = 0.21, T/C JM221 = 0.10. Further, several compounds are less emetogenic when administered orally to the ferret, compared with CDDP given iv. It is concluded that the platinum IV ammine/amine dicarboxylates studied are representatives of a novel class of antitumour drugs which are capable of oral administration and which provide an important lead to the circumvention of cisplatin resistance.

BACTERIAL EXPRESSION OF SPECTRALLY ACTIVE RAT CYTOCHROME P-450 2G1 (P-450 olf)

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Cytochrome P-450 catalyzes the oxidation of a variety of chemicals, both natural and synthetic. Numerous isozymes have been isolated and characterized by various means. We have recently described a unique isozyme, P-450 CYP2G1 (1) expressed exclusively in olfactory mucosa. It's function and catalytic properties remain unknown, largely due to the scarcity of tissue and unavailable pure protein. We therefore expressed CYP2G1 cDNA in bacteria to obtain large amounts of the pure protein by biotechnology. The cDNA was shortened by 99 base pairs at the 5' amino terminus and inserted into the bacterial expression vector pDS. Transformed bacteria were induced to express protein with IPTG in the presence of the heme precursor, δ-amino levulinic acid. Spectral analysis revealed a substantial amount of spectrally active P-450 in E.coli. The spectrum was characteristic for P-450, exhibiting a ferrous CO spectrum at 450nm with virtually no absorption at 420nm. This indicates the presence of an active P-450 produced in these cells. Furthermore typical P-450 binding spectra with metyrapone and testosterone were observed. Immunochemical analysis revealed a protein with identical MW to the protein transiently expressed in mammalian COS-1 cells from the same cDNA. A number of odorant substrates are presently tested with this olfactory P-450.

In conclusion, bacterially expressed P-450 provides a source of large quantities of protein with identical characteristics of the mammalian product. This recombinant protein can, in turn, be used to assess the metabolic activity of uncharacterized and low abundant P-450's. In addition, a large source of pure protein forms the basis for attempting crystallization of the protein.

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DISPOSITION OF CADMIUM DURING CADMIUM TOLERANCE

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An intriguing phenomenon observed in animals pretreated with Cd is development of tolerance to a subsequent dose of the metal. Possible mechanisms for this tolerance include reduced absorption, an altered tissue distribution, and an altered subcellular distribution of Cd. Male rats received a single Cd pretreatment (2.0 mg/kg, sc) 24 hr prior to administration of a typically lethal challenge dose of Cd (3.9 mg/kg, iv). Tolerance was evident because no mortality was observed in Cd-pretreated rats. Because Cd induces synthesis of hepatic metallothionein (MT), a higher percentage of the challenge dose might be sequestered in the liver of Cd-pretreated animals with less distributed to target organs of toxicity. At 2 and 24 hr following Cd challenge, no marked changes in organ distribution of the challenge dose of Cd were observed as a result of Cd pretreatment. In addition, no increase in biliary excretion of Cd was observed in the tolerant animals, in fact a marked decrease was observed. However, isolation of hepatic subcellular fractions 2 hr following injection of the challenge dose revealed less Cd in nuclei, mitochondria, and endoplasmic reticulum, and more in cytosol as a result of Cd pretreatment. The increased cytosolic Cd was bound primarily to MT which had been induced markedly by Cd pretreatment. These data indicate that differences in absorption or tissue distribution of Cd are unlikely explanations for development of tolerance to Cd. Rather, tolerance appears to result from an MT-related change in the hepatic subcellular distribution of Cd, evidenced by lower concentrations of Cd in nuclei, mitochondria, endoplasmic reticulum, and cytosolic high-molecular-weight proteins and higher concentrations bound to MT in cytosol. (Supported by NIH Grant ES-01142)

MECHANISMS OF IDIOSYNCRATIC DRUG REACTIONS : ABOUT THE ORIGIN OF IMMUNOALLERGIC DRUG-INDUCED HEPATITIS.

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Drug-induced immunoallergic hepatitis remain a very serious problem for pharmaceutical companies, as their occurence cannot be predicted by usual toxicological tests. In order to find animal predictive tests for such toxic effects, it is necessary to understand their detailed mechanisms. The strategy that we have followed for that was to use the specific anti-tissue auto-antibodies which appear in some cases. In that context, we have recently showed that the anti-LKM_2 antibodies appearing in tienilic acid-induced hepatitis are directed against a human liver cytochrome P-450 of the IIC subfamily, whereas the anti-M_6 antibodies appearing in iproniazid-induced hepatitis are directed against liver mitochondria monoamine oxidase B[1,2]. This led us to propose a detailed mechanism for these drug-induced hepatitis, where the enzyme involved in the activation of the drug is alkylated during this reaction and becomes immunogenic. However, at least two main questions remained unanswered : a) what is the nature of the reactive metabolite responsible for protein alkylation ? b) What is the nature of the alkylated protein and where is it alkylated ?

A possible approach to answer these questions will be presented, and recent results will be given in the particular case of tienilic acid. This approach consists in the use of human liver cytochromes P-450 of the IIC subfamily expressed in yeast. It allowed us to better determine the human P-450 isoenzyme mainly involved in the 5-hydroxylation and metabolic activation of tienilic acid in human liver, and to obtain first data in the search of the nature of the electrophilic reactive intermediates formed by metabolic oxidation of this drug and responsible for its covalent binding to liver proteins.

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HEPATIC TRANSPORT MECHANISMS

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Introduction. The liver contains at least four different cell types for the disposition of drugs and (glyco-)proteins: parenchymal cells (hepatocytes), Kupffer cells (macrophages), endothelial cells and lipocytes. These cells are differently equipped to take up, degrade and/or excrete drugs and macromolecules. Among these processes are carrier-mediated membrane transport, adsorptive endocytosis, receptor mediated endocytosis, exocytosis or diacytosis as well as various metabolic and proteolytic routes. Knowledge of these processes is necessary for understanding non-linear kinetic patterns, drug interactions, drug toxicity as well as for drug delivery to the organ.

The hepatocytes take up and secrete drugs with a proper balance of hydrophilic and hydrophobic properties. This is due to specific properties of the carriers at sinusoidal and canalicular level as well as the ability of the liver to extract highly protein-bound agents from the bloodstream. Neo-glycoproteins can be manifactured such that specific uptake in the various cell types occurs. This concept can be used for cell-specific targeting of antiviral drugs and antitoxicants and even for in vivo delivery of genes to correct genetic deficiencies.

Periportal (zone 1) and pericentral (zone 3) cells are differently involved in uptake and further disposition of drugs and macromolecules. This can be due to sinusoidal concentration gradients, to intrinsic cellular differences in carrier- and receptor mediated transport. Zonal differences in site of metabolite formations, unequal plasma protein binding and carrier-affinity at both poles of the cell explain the typical differences in sulfate- and glucuronide-drug conjugate excretion into blood and bile respectively.

Classical theory postulates separate transport mechanisms for cationic, anionic and uncharged drugs in the liver. However, within each of these categories at least four sub-mechanisms should be postulated. Overlapping substrate specificity and the presence of a non-specific uptake system explain and predict major interactions of drugs and their metabolites in the liver. Driving forces for membrane transport were studied and photoaffinity labeling, isolation and reconstitution of transport systems were performed to further characterize these processes. Rate-limiting steps such as sinusoidal uptake and efflux, subcellular sequestration and canalicular extrusion determine the relative contribution of the liver in distribution and elimination of drugs and drug metabolites in the body as well as local toxicity within the organ.

GENOTOXICITY AND DNA-BINDING OF NEUROTOXIC ORGANOPHOSPHATES

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2-(o-Phenyl)-4-1:3:2-benzodioxaphosphoran-2-on (PBD) is an electrophilic and neurotoxic metabolite of triarylphosphates. We have investigated the genotoxicity of this saligenin phosphate and the structure of DNA-adducts formed. PBD was a potent mutagen in the Ames-test (875 rev./µmole, *S. typhimurium* TA100) which did not require metabolic activation. PBD also induced chromosomal damage indicated by dose-dependent formation of micronuclei in Syrian hamster embryo fibroblasts. Incubation of PBD with guanosine and cytidine resulted in formation of guanosine- and cytidine-adducts which were identified as N-2-(o-hydroxybenzyl)guanosine (I) and O^6-(o-hydroxybenzyl)cytidine (II) by ^{1}H-NMR-spectrosopy, thermospray mass spectrometry and pH-dependent electronic spectrometry. The formation of (I) and (II) was also demonstrated when PBD was incubated with calf thymus DNA, it is due to a p-hydroxybenzylation from cyclic phosphoranes derived from o-alkyl substituted triarylphosphates. The obtained results indicate that some triarylphosphates, besides their neurotoxic effects, may have a carcinogenic potential.

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THE METABOLISM AND EXCRETION OF RISPERIDONE IN RATS, DOGS, AND MAN.

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Risperidone (RIS) is a novel antipsychotic drug. Its main indication is the maintenance treatment of chronic schizophrenia.
For metabolism studies, ^{14}C-RIS and ^{3}H-RIS were used, labelled in different parts of the molecule. After a single oral dose in male and female rats, the radioactivity was excreted predominantly in the faeces (78-82% of the dose) and for only 14-20% in urine. Biliary excretion of the radioactivity amounted to 69-78% of the dose. Only about 15% of the biliary radioactivity was subjected to enterohepatic circulation. In orally dosed dogs, the fractions of the radioactivity recovered in the urine (44-50%) and faeces (40-42%) were similar. In volunteers, within 7 days after a single oral dose about 70% was excreted in the urine and 12-18% in the faeces.

Despite the large species differences for the fractions of the dose excreted in urine and faeces, the metabolite patterns in the excreta of rats, dogs and humans were qualitatively very similar. The main metabolic pathways of risperidone in the three species were the (alicyclic) hydroxylation in the 9- and 7-positions of the tetrahydro-pyrido-pyrimidinone moiety and the oxidative *N*-dealkylation at the piperidine nitrogen. In faeces, there was a large fraction of metabolites with an opened benzisoxazole ring.
The mass balance of the metabolites of RIS in man was similar to that in dogs; in rats, the metabolism was more extensive.

9-Hydroxyrisperidone (9-OH-RIS) was the main plasma metabolite in the three species. The pharmacological profile and potency of 9-OH-RIS is similar to that of RIS. In humans, the formation of 9-OH-RIS is subject to the debrisoquine-type genetic polymorphism.

N-ACETYLTRANSFERASES IN MAN

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Acetylation by arylamine N-acetyltransferases (NATs) is a major route in the metabolism of numerous drugs and procarcinogens. A genetic polymorphism of clinical relevance divides the population into slow and rapid acetylators of several arylamine drugs. Two NATs, NAT1 and NAT2, have recently been characterized by protein purification and by cloning and functional expression of the respective genes, which both were localized to chromosome 8. NAT1 codes for a protein with ubiquitons tissue distribution and a high affinity for p-aminobenzoic acid and p-aminosalicylic acid, so-called monomorphic substrates. NAT2 codes for a protein predominantly expressed in liver with a high affinity for sulfamethazine and other polymorphically metabolized drugs. NAT2 was analyzed at the level of protein, RNA and DNA derived from phenotyped slow and rapid acetylators. Two common (M1, M2) and one rare (M3) mutant allele were identified and their mutations characterized. A simple PCR-based DNA test can identify > 95 % of mutant alleles and predict the phenotype.

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SUBSTRATE AND INHIBITOR PROBES FOR HUMAN CYTOCHROMES P-450

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Cytochrome P-450 1A2 and isozymes of the 2C subfamily are known to be of importance in drug and chemical metabolism and ongoing studies in this laboratory have focussed on the characterisation of probes suitable for application to studies defining the xenobiotic substrate profiles and regulation of these enzymes.

The primary metabolic pathway of caffeine (CA) is 3-demethylation to form paraxanthine (PX). It has been suggested that this reaction is mediated by P-450 1A2, but kinetic studies in human liver microsomes demonstrated the involvement of at least two isozymes in PX formation. The high affinity component (Km 0.2 mM) of CA 3-demethylation was inhibited by alphanaphthaflavone (IC50 0.25μM) whereas the low affinity component (Km 30mM) was unaffected. An expressed P-450 1A2 cDNA converted CA to PX in a manner similar to the high affinity component of CA 3-demethylation in human liver microsomes. Thus, CA serves as a probe for P-450 1A2 only at low substrate concentrations (<0.1mM). The minor pathways of CA metabolism may also be used as probes for other human P-450s.

The hydroxylations of tolbutamide and phenytoin are mediated predominantly by a single P-450. An expressed human P-450 2C9 cDNA was shown to catalyse the hydroxylations of both tolbutamide and phenytoin. The kinetic and inhibitor characteristics of the expressed enzyme were comparable to those of the human liver microsomal tolbutamide and phenytoin hydroxylase activities, indicating 2C9 may well be the isozyme primarily responsible for the metabolism of these drugs in humans.

SENSITIVE ANALYTICAL METHOD FOR A DRUG AND ITS METABOLITES USING A BIOIMAGE ANALYZER — DIAZEPAM AND ITS METABOLITES IN PREGNANT AND FETAL RATS

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Bioimage Analyzer (BA) system is a two-dimensional radioactivity detector based on an imaging plate which is several hundred times more sensitive than X-ray films. Its dynamic range and linearity are also far better than X-ray films. We aimed to apply the BA system to the determination of drug and its metabolites concentration in biological samples using ^{14}C-diazepam and thin-layer chromatography (TLC). The system showed a good linearity within 10 to 10,000dpm in ca. 1cm^2 spot on a TLC plate. This sensitivity was high enough to perform quantitaive analyses of diazepam and its metabolites in fetal rat tissues.

After i.v. injection of ^{14}C-diazepam at a dose of 0.3mg/55μCi/kg to pregnant rats, the concentrations of diazepam and its metabolites in maternal plasma, fetuses and amniotic fluid were analyzed at 13, 16 and 19 days of gestation. The results showed the presence of placental transfer of phase II metabolites of diazepam in pregnant rats. At the late stage of pregnancy, phase II metabolites of diazepam in fetuses were mainly eliminated to the amniotic fluid resulting in a higher concentration ratio of phase II metabolites in amniotic fluid v.s plasma of pregnant rats.

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XENOBIOTIC METABOLISING ACTIVITY OF HUMAN AND RAT EPIDERMAL KERATINOCYTE CULTURES

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The use of human and rat epidermal keratinocyte cell cultures may prove most useful in providing metabolic data which cannot be adequately obtained by other *in vitro* methods. We have successfully cultured epidermal cells from adult human skin and rat neonatal skin, using mitomycin-treated 3T3 cells as a feeder layer. Drug metabolising activities of these cultures have been investigated.

At the monolayer stage, after day 9 of culture, the human and rat epidermal cultures express measureable levels of 7-ethoxycoumarin O-deethylation (ECOD) (1.3-8.8 pmol hydroxycoumarin/min/10^6 human cells; 1.6-9.2 pmol hydroxycoumarin /min /10^6 rat cells) and 7-ethoxyresorufin O-deethylation (EROD) (0.5-0.7 pmol resorufin/min/10^6 human cells ; 0.4-0.7 pmol resorufin/min/ 10^6 rat cells), indicating the presence of mixed function oxidase activity in the cells. These activities continue to be expressed up to 26 days in culture, and represent 0.4-2.6 % ECOD and 2.9-5.5 % EROD values of isolated rat hepatocytes.

Other metabolic activities have been detected in the cultures including esterase activity (towards benzyl acetate), alcohol dehydrogenase activity (towards benzyl alcohol and cinnamaldehyde) and aldehyde dehydrogenase activity (towards cinnamaldehyde). High concentrations of reduced glutathione have also been detected in both rat and human epidermal cultures (21.5±5.5 nmol/10^6 human cells; 17.5±10.5 nmol/10^6 rat cells) which are similar to those found in isolated rat and human hepatocytes.

These studies were supported by a grant from the UK Health and Safety Executive (no.1/LMD/126/212/89).

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CYTOCHROMES P450: ACTIVE SITES AND SAR

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In the absence of crystallographic or other direct structural data, efforts to characterize the active sites of cytochrome P450 enzymes depend on indirect evidence and inferences made from comparisons with the structure of cytochrome $P450_{cam}$ (P450 CIA1), the only isozyme for which a crystal structure is available. In order to place such structural comparisons on a firm footing and to define the factors that control substrate specificity, we are carrying out parallel studies of the catalytic chemistry of cytochrome $P450_{cam}$ and several rat liver isozymes, including P450IIB1 ($P450_b$) and P450IA1 (P450c). The absolute regio- and stereochemistry of the oxidation of styrene and substituted styrenes by these enzymes shows that cytochrome $P450_{cam}$ is more stereoselective than cytochrome P450IIB1. Molecular dynamics calculations carried out with Dr. Gilda Loew and her coworkers correctly predict the stereochemical specificity of the $P450_{cam}$-catalyzed oxidation of at least some unsaturated substrates completely unrelated to camphor. The reactions of the active sites with heme-modifying reagents also permit structural comparisons to be made between the bacterial and hepatic isozymes and inferences to be made concerning the active sites of the latter. This work is supported by NIH grants GM25515.

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MECHANISMS OF IDIOSYNCRATIC DRUG REACTIONS

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Adverse drug reactions which cannot be predicted from the known pharmacological properties of the drug and which are not easily reproduced in experimental animals are a major complication of drug therapy. It is necessary to investigate the mechanisms of such toxic reactions in order to 1) define structural features within a given drug which are responsible for causing toxicity and 2) to help identify those individuals who are particularly sensitive to a given drug reaction. Adverse reactions may arise because of immune-mediated tissue damage and by direct toxicity caused by either the parent drug or a metabolite. Therefore both the rate and the route of drug metabolism may influence drug toxicity. The balance between bioactivation and detoxication pathways will be a crtical factor in cell-directed toxicity.

We have therefore developed *in vitro* techniques that incorporate human cells and tissues for the detection and characterisation of stable, chemically reactive and cytotoxic metabolites. In addition the chemical mechanism of drug antigen formation can be elucidated.

The relevance of *in vitro* experiments to drug toxicity observed in man will be illustrated by reference to studies with anticonvulsants, antidepressants and antimalarials.

DISPOSITION OF ORGANOTIN COMPOUNDS

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Within the past 10-15 years numerous studies have been conducted with several di- and trisubstituted organotin compounds. Industrially they are mainly used as heat stabilizers for polyvinyl chloride polymers and as agricultural biocides, respectively. These studies have indicated that for some of the di- and trialkyl substituted homologues the immune system, in particular the thymus, is the most sensitive target in rats. (1) However, besides unsolved interspecies differences also differences in immunotoxic effects are observed depending the route of administration of di- and triallyltins (oral versus intravenous), and the length of the alkyl chain.
In this paper the absorbtion, tissue distribution and excretion of 14C- labeled di-n-octyltin dichloride (DOTC) and 14C- labeled tri-n-butyltin acetate (TBTA) in rats will be presented and discussed after both oral and intravenous application. In addition, since tri-n-butyltin chloride was found to be about 40% less active than di-n-butyltin dichloride with respect to induction of a similar thymus atrophy the metabolism of tributyltins to dibutyltins, the latter suspected to be the ultimate immunotoxic compound, was studied. In this respect both the route of administration and the relative importance of liver versus gastrointestinal metabolism will be discussed.

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IDIOSYNCRATIC ALLERGIC HEPATITIS

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It has been estimated that more than 600 drugs may cause hepatic injury. Drugs that cause hepatotoxicity have been classified into two categories; those that are intrinsic hepatotoxins and those that are idiosyncratic hepatotoxins. The hepatotoxicity caused by intrinsic hepatotoxins, such as chloroform and acetaminophen, is generally characterized as being host independent, dose dependent, and reproducible in animals. The majority of drugs that cause hepatotoxicity, however, appear to be idiosyncratic hepatotoxins. Their reactions are host dependent, often apparently dose independent, and difficult to reproduce in animals. They are also hard to predict and usually are not recognized as a potential medical problem until the drug has been in general use for some time. It is currently believed that most idiosyncratic drug-induced hepatotoxicities are due to allergic or hypersensitivity reactions. For example, patients diagnosed with halothane hepatitis have been shown to be sensitized to several liver proteins that are covalently modified by the trifluoroacetyl chloride metabolite of halothane. Recently, several of these proteins have been purified and identified. They are a 100 kDa protein that is GRP94 (glucose-regulated protein), a 82 kDa protein that is ERp72 (endoplasmic reticulum protein), a 80 kDa protein that is GRP78, a 59 kDa protein that is carboxylesterase E1, ES-8/ES-10, and a 57 kDa protein that is protein disulfide isomerase. In addition, a 58 kDa protein also has been purified that does not appear to correspond to any known protein.

METABOLISM OF XENOBIOTICS IN THE ENVIRONMENT: DIOXINS AND DIBENZOFURANS

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The persistence of chlorinated dioxins and dibenzofurans in the environment and the biosphere is essentially determined by their physicochemical properties and metabolic stability. Out of the 210 congeners released from various sources only a selected group with common structural characteristics is usually present in biological material. These congeners carry at least four chlorines at the lateral (2,3,7,8-) positions and represent the most potent members of both groups, regarding biological activity and toxicity. Their accumulation in the human food chain has raised public concern.

The halogen substitution pattern plays a key role in the biological fate of the individual congeners: Those having adjacent unsubstituted carbons are metabolised efficiently by procaryotic or eucaryotic enzyme systems. In contrast, 2,3,7,8-TCDD and other laterally substituted congeners are very resistant to biodegradation. Their long persistence in soil reflects the inability of the microflora to use such compounds as substrates. In experimental animals, 2,3,7,8-TCDD and other toxic dioxins and dibenzofurans are metabolized slowly. Structure analysis of some of the hydrophilic derivatives has shown that hydroxylation and reductive dehalogenation, as well as conjugation reactions occur in the liver. The polar metabolites are rapidly excreted and exhibit no dioxin-like activity. Recent investigations on a brominated analogue indicated similar metabolic routes. The biotransformation rate of higher chlorinated (2,3,7,8-substituted) congeners depends on the position of the additional substituents and generally decreases with increasing chlorination.

Clearance of 2,3,7,8-TCDD and other toxic congeners from the human body is markedly slower than from rats and other species. Obviously, human cytochrome P450 is rather inefficient for degrading this type of compounds, a fact which needs to be considered in human risk assessment.

This paper will focus on the present status of knowledge and emphasize the significance of kinetics, determining toxicity.

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FATE OF XENOBIOTICS IN SOIL : SAFETY EVALUATION

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The fate and behaviour of pesticides and other organics in soil continues to receive increased attention. As pesticides are designed and registered for use in agricultural environments, a great deal is known about the fate in soil of this group of chemicals. Particular emphasis is placed on determination of persistence or "half life" and degradation pathways of individual pesticides and on mobility leading to possible presence in surface and ground waters.

Pesticides and other organics can potentially undergo biodegradation, chemical and/or photochemical conversion and basic information on transformation processes and products formed is obtained from laboratory studies using soils or occasionally microbial isolates. However, only under outdoor conditions can the relative importance of these various processes be assessed. Similarly, laboratory soil leaching tests provide information on the relative mobility of compounds, and lysimeter or field studies are necessary to assess mobility with natural soil profiles and climatic conditions.

Typically a sequential approach is followed but approaches to the regulatory evaluation of pesticide degradation and mobility in soil vary from country to country, with differences in experimental approaches and guidelines. For example, although most major regulatory authorities require basic laboratory studies, albeit differing in detail, lysimeter studies can be required in Germany and Scandinavia, field studies are preferred in the USA, and computer simulation modelling is a favoured approach in The Netherlands. These various approaches will be discussed together with the relevance to other organic chemicals.

HEPATOCYTES AS BIOTRANSFORMATION MODEL

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During the last few years alternative in vitro models for biotransformation studies of xenobiotics have gained popularity, not only because of the increasing criticism on in vivo experiments but also because of improved technology and the existence of a number of economic, scientific and ethical advantages proper to these in vitro techniques. It is generally accepted that freshly isolated hepatocytes of various species (including man), kept in suspension for some hours, represent a suitable model for biotransformation, acute toxicity and mechanistic studies of xenobiotics.

For the investigation of long-term effects, primary monolayer cultures of hepatocytes and their co-cultures with rat epithelial cells, derived from primitive biliary duct cells, have been proposed. None of both systems is ideal but the biochemical and morphological integrities of the latter model are better kept as a function of time.

An overview of experimental results obtained using pure cultures and co-cultures of adult rat hepatocytes, kept under various culture conditions will be presented.

Phase I biotransformation parameters such as cytochrome P-450, its isoenzyme pattern and key enzymes in biotransformation will be considered in both in vitro models. Concerning phase II, glutathione S-transferase and its isoenzyme pattern will be discussed.

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FOOD CONSTITUENTS AND INTESTINAL MICROBIAL DRUG METABOLISM

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Intestinal microbial metabolism of drugs and other xenobiotics in mammals is confined mainly to the large bowel where the greatest number of microorganisms reside, particularly in man. Consequently the drugs most likely to interact with the gut microflora are those which are poorly absorbed after ingestion or which are secreted into the intestine as conjugates in bile.

Although diet does not appear to have a major influence on the types of bacteria present in the gut, there is ample evidence that bacterial metabolic activities are altered by dietary modification. Among food constituents, changes in the type and level of dietary fibre and fat have the greatest influence on the gut microbial metabolism of xenobiotics. In the case of dietary fibre, the effects depend greatly on the fermentability of the material - poorly fermentable fibre sources tend to depress metabolism whereas highly fermentable fibres can stimulate microbial activity by providing a source of energy for gut bacteria. For example, in rats, dietary carrageenan (a poorly fermented fibre source) strongly suppresses reduction of the nitro group of drugs such as metronidazole and nitrofurantoin by the gut microflora. More fermentable fibres, such as pectin and guar gum, tend to increase nitroreduction by the gut flora, and may also modify microbial metabolism via reducing colonic pH.

* Increasing the fat content of the diet markedly increases the rate of hydrolysis of glucuronide conjugates by intestinal bacteria, thus potentially increasing the enterohepatic circulation of drugs.

HALOGENATED AROMATIC COMPOUNDS AS CYTOCHROME P-450 INDUCERS

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds induce rodent hepatic cytochromes P-4501A1 and P-4501A2 *in vivo*; however, in transformed mammalian cells in culture only the former protein is induced. The molecular mechanism of TCDD-induced CYP1A1 gene transcription has been extensively investigated and involves the initial binding of TCDD to the cytosolic Ah receptor, a transformation step followed by nuclear translocation and interaction of the occupied nuclear receptor complex with genomic dioxin response elements (DREs) in the 5'-flanking region of the gene. The structure-dependent induction of CYP1A1 gene expression by polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) is consistent with the role of the Ah receptor; however, the structure-dependent steps in the induction process have not been established. Using a series of radiolabeled and unlabeled PCDD and PCDF congeners with different induction potencies, the molecular basis for the structure-dependent induction of CYP1A1 gene expression has been investigated. The results indicate that for these compounds, the rate of accumulation of occupied ligand-Ah receptor complex in rat hepatoma H-4-II E cells correlates with the relative induction potencies of these congeners. Moreover, using rat cytosol it was also apparent that the rates of transformation of the cytosolic receptor-ligand complexes to forms which retard the mobility of a ^{32}P-labeled consensus DRE were also dependent on the structure of the ligand. These results, coupled with the ligand-dependent initial formation of the cytosolic Ah receptor complex, indicate that the structure-dependent induction of CYP1A1 gene expression is due to differential ligand-receptor interactions in this multi-step process. (Supported by the National Institutes of Health ESO-3554).

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PEPTIDE METABOLISM

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The search for therapeutic peptides and the investigation of their metabolic fate present specific challenges for the pharmacologist and biochemist. In studies on quantitative structure-activity relation (QSAR) for selection of peptide analogues, enzyme resistance in vitro is often a useful parameter of selection. However, the nature of peptidase enzymes which inactivate a compound in vivo is in most instances poorly understood at the beginninng of an investigation, and different accessibility to compartments in the body may change or mask the local rate of inactivation. Analytical detection of peptide metabolites has greatly improved over the last decade, especially for peptide analogues. They frequently incorporate characteristic chemical groups which facilitate detection by HPLC/RIA (in the therapeutic dose range), or HPLC/betaspectrometry for labels which serve as markers for organ distribution and metabolism studies (in the analytical dose range). Local metabolism is increasingly of interest for new dosage forms (e.g. nasal and buccal route), whereas general metabolism e.g. by liver and kidney (parenchymal cells) or lung (endothelial cells) remains the decisive factor for finding peptide analogues with prolonged duration of action and better absorption. Starting from organ distibution studies and perfusion of isolated organs, an effective approach to improving enzyme resistance can be found in QSAR studies. Comparative metabolism in several animal species is helpful, although differences between species tend to be minor. For therapeutic peptides, receptor-associated enzymes may be of some importance and the specific receptor preparations used by pharmacologists should be explored accordingly whether specific peptidase enzymes can be identified for the ligand. Finally, the residual biological (pharmacological) activity of potential metabolites provides valuable information at early stages of QSAR studies, and is likely to provide insight together with receptor binding studies when obvious cleavage sites are present which could be protected without interfering with binding.

INTESTINAL METABOLISM OF *SACCHAROMYCES BOULARDII*, A MODEL ORAL BIOTHERAPEUTIC AGENT.

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Saccharomyces boulardii (Sb) is a nonpathogenic yeast used in the treatment of antibiotic-associated diarrhea and pseudomembranous colitis. It is not absorbed orally but does survive GI transit. Animal studies have shown that the microbial content of the GI tract has a significant effect on Sb survival. We investigated the effect of antibiotic(s) on the metabolism of Sb in the GI tract.

Rats were given single or daily oral doses of Sb and the amount of viable yeast in stools was quantitated. Sb was eliminated exclusively in the feces with less than 3% of the oral dose recovered from rats not receiving antibiotics. Treatment with clindamycin or ampicillin, agents active against anaerobic bacteria, resulted in a significantly higher recovery of the administered dose (8% and 14%, respectively). These antibiotics also decreased elimination rates ($T_{1/2}$=7.1 and 7.2h, respectively) compared to untreated controls ($T_{1/2}$=2.6h). The ampicillin and clindamycin-induced kinetic changes were also observed in animals in which fecal steady state yeast levels were achieved by daily oral dosing.

In a human study, 11 volunteers were given a single oral dose of Sb, and viable yeast levels in stools was quantitated. It was found that 8/11 cleared the single Sb dose within 3 days. Ampicillin treatment dramatically increased the recovery of Sb. In some cases, a 20- to 78-fold difference between pre- and post-ampicillin Sb recoveries was observed. Sb was cleared more slowly (4-5 days) in 3/11 volunteers, accompanied by a relatively lower ampicillin effect.

Collectively, these results show that the Sb-antibiotic interaction can markedly influence the level of Sb in the GI tract. This can presumably be attributed to an antibiotic perturbation of the normal destructive processes of the intestinal microflora. Sb is therefore a useful model for the study of the GI metabolic destruction of orally administered biotherapeutic agents.

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CHARACTERIZATION OF MOLECULAR SPECIES OF LIVER MICROSOMAL CARBOXYLESTERASES OF SEVERAL ANIMAL SPECIES AND HUMANS

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Carboxylesterases(EC 3.1.1.1) are well recognized to be important for the activation of prodrugs and inactivation of various xenobiotics. We purified fifteen isozymes of carboxylesterases from liver microsomes of ten animal species and humans(1,2), and their physical, enzymological and immunological properties were compared each other. The carboxylesterase isozymes tested have similar subunit weight(57-64KDa), but pI values are differ widely (4.7-6.5). The amino acid composition of carboxylesterase isozymes showed considerable similarities. In addition, th amino-terminal amino acid sequences showed a striking homology, though the first amino acid itself in the sequence is different in all the isozymes tested. Rat RL2 which is one of the three isozymes of rat liver carboxylesterases was found to be identical to egasyn which is accessory protein of B-glucuronidase in liver microsomes. On the other hand, some carboxylesterase isozymes are involved in lipid metabolism in terms of acyl CoA hydrolase, acyltransferase and monoglycerol lipase activities.

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CONCERNS IN THE EVALUATION OF THERAPEUTIC RECOMBINANT PROTEINS

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Recombinant technology has provided the means to produce therapeutic drugs and biologicals that had previously been obtainable only in small quantities. In contrast to conventional drugs which have been primarily small molecules produced by organic synthesis, these therapeutic products are produced in living organisms. Biological synthesis necessarily invokes diversity and, therefore, biological products are composed of heterogeneous mixtures of active species that have been historically difficult to characterize. Although recombinant DNA technology has allowed the production of large quantities of therapeutic proteins in cell culture, it has not eliminated the heterogeneity and diversity that is associated with biological products. The heterogeneity and diversity which define a biological product are determined by the process of manufacture. This process includes the nature of the host cell, the conditions of host cell propagation, the purification strategy, and the formulation steps. All of these steps contribute to a biologicals' diversity. Therefore the careful evaluation of the manufacturing process in addition to the recombinant protein is essential to the review of a biological for use as a therapeutic or a prophylactic product such as a vaccine. The above considerations will be discussed with regard to a diverse group of biological products and production systems.

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TRANSPORT MECHANISMS IN THE SKIN

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Anatomically the human skin can be described as a laminate structure: the stratum corneum/epidermis as the outermost layer, and the viable dermis as underlying tissue. There are also shunt pathways through the skin: hair follicles, sweat glands and sebaceous glands.

The stratum corneum consists of a layer of dead cells that acts as a passive solution-diffusion membrane that resists mass transport to a much greater extent than the dermis for most drugs, except the most hydrophobic substances. Therefore, once drugs traverse the epidermis they are readily taken up by the capillary circulation. Permeation enhancers can be used to significantly increase passive transdermal transport. These substances appear to act most importantly on the solubility of the drug in the skin, rather than on the diffusion coefficient.

The shunt pathways cover only a fraction of the total skin surface area and are not important for most passive transport processes except for very hydrophilic drugs or when an electrical field is added as a driving force. In this latter case, called electrotransport, drug mass transport occurs through the shunts by electro-diffusion and electro-osmosis. Compared to passive transdermal transport, the electrotransport mode of drug delivery can provide significantly higher mass transport rates even for higher molecular-weight drugs.

This presentation will address both passive and electrotransport drug delivery processes as they relate to drug metabolism.

XENOBIOTICS AND METABOLITES : BINDING TO BLOOD COMPONENTS.

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Xenobiotics and drugs usually exhibit multiple binding in blood to cells, platelets or plasma proteins (albumin, alpha$_1$-acid glycoprotein, lipoproteins). It is important to know which are the relevant bindings on either a pharmacokinetic or a pharmacodynamic point of view, and how the bindings can be modified by pathological states. First, drug binding in blood may impair, permit or improve the drug transfer across cell membranes, as can be deduced from the apparent drug distribution volume or from experimental *in vivo* methods (Oldendorf's technique). Second, some binding in blood can occur on circulating receptors close to the tissue receptors, and this binding may be a probe or an image of tissue receptor occupation, i.e., tissue effects. This is particularly interesting when there is no other way to measure drug effects, and it has been suggested to monitor for example beta-agonists via lymphocytes beta-receptors, cyclosporine A via lymphocytes T and colchicine via leucocyte concentrations. Finally, some pathological states can modify the drug distribution among blood carriers and may involve some change in the drug availability for tissues. The most known example is that of albumin-bound acidic drugs which are displaced by high concentrations of bilirubin. Most of the characteristics of xenobiotics blood binding may be assessed before any clinical trial with *in vitro* and *ex vivo* experiments.

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DETERMINATION OF RATES OF HYDROLYSIS OF VASOPRESSIN ANALOGS BY DIGESTIVE PROTEASES USING AN IMMOBILIZED DIGESTIVE ENZYME ASSAY.

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An in vitro assay using immobilized digestive proteases has been developed to predict the relative rates of hydrolysis of orally administered peptide drugs and drug analogs by the digestive system. Lysine vasopressin, arginine vasopressin, and deaminocysteine-D-arginine vasopressin were incubated in vitro with three sets of immobilized digestive enzymes which simulated gastric digestion in the stomach, digestion by pancreatic enzymes in the intestinal lumen, and digestion by mucosal peptidases at the intestinal brush border. Rates of hydrolysis of each peptide substrate by each set of enzymes were determined by monitoring the disappearance of the starting material using continuous-flow fast atom bombardment (FAB) mass spectrometry with selected ion monitoring. Continuous-flow FAB liquid chromatography mass spectrometry was also used to identify the hydrolysis products of each enzymatic reaction. Data on the relative stabilities of the vasopressins measured using the in vitro digestive enzyme assay were compared to results obtained in vivo using a rat bioassay. The vasopressins that showed the highest activity in the rat bioassay following oral administration were also the most stable to proteolysis in vitro. This assay could be useful as a rapid technique to select the most stable peptide analog drugs for further development, and for the formulation of these drugs for oral administration. Finally, the application of this method should reduce the number of vertebrate animals used in drug development.

THE ROLE OF MASS SPECTROMETRY IN DRUG METABOLISM STUDIES

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The coupling of separation methods and mass spectrometry in particular liquid chromatography (LC-MS) has been an important development in the field of drug metabolism. LC-MS systems have been realized by various different approaches and several commercial interfaces are available such as particle beam, thermospray, continuous flow FAB and electrospray. Although compounds can be analyzed nowadays for a broad range of polarity, this does not automatically mean that the LC/MS approach can be realized in a straightforward manner and directly applied in metabolism studies and trace analysis.

Mass spectrometric detection offers high selectivity but often the overall selectivity is insufficient and tandem mass spectrometry is necessary. For qualitative analysis tandem mass spectrometry is mandatory since most ionization methods used in LC/MS are "soft" and do not generate sufficient structural indicative fragment ions.

For polar compounds other problems occur in LC/MS arising from the fact that chromatographic systems are used for this class of compounds with mobile phases containing non-volatile buffers or additives such as ion-pairing agents. These systems are not directly compatible with mass spectrometric detection. However, using simplified coupled column chromatography approaches, such as the phase system switching method, improve the compatibility by decoupling of the separation step and the detection step while the optimum parameters for both stages can be chosen.

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BIOMONITORING OF INDUSTRIAL CHEMICAL AND AGROCHEMICALS

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In industry, biological monitoring (BM) is an important component for health risk assessment of chemical exposure. Using this technique the integral dose of a chemical absorbed into the body via all routes of exposure is assessed. BM is particularly of interest if skin exposure is a main route of exposure, such as may be the case during field application of agrochemical formulations but also during manufacture or use of many industrial chemicals. In addition the technique can be efficiently used to measure low levels of internal exposure to potentially carcinogenic or mutagenic chemicals.
In addition to the measurement of internal chemical exposure, use of the technique may reveal differences in toxicokinetic parameters between human beings but also between humans and experimental species.

BM can only be applied if suitable markers of internal exposure have been identified which usually occurs in the study of the biotransformation of xenobiotics in experimental species. The applicability of identified metabolites for biomonitoring of course depends on the similarity of biotransformation pathways in humans and the experimental species.

During this presentation practical examples of recent findings on BM of occupationally exposed workers will be presented. The human surveillance data of the chemicals will be reviewed in the order of predictive value for health risk assessment.

CONJUGATIVE METABOLISM IN VIVO

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Several drugs undergo direct glucuronide and/or sulfate conjugation usually leading to inactive conjugates. Therefore, factors affecting the rate of glucuronidation-sulfation also influence the intensity of duration of the pharmacologic effect of such drugs. We used diflunisal (DF), a compound forming two types of glucuronides (a phenolic (DPG) and acyl (DAG) glucuronide) and a sulfate (DS), as a model compound to study various factors affecting in vivo glucuronidation-sulfation. In healthy volunteers a marked decrease in CL_{DPG} and CL_{DAG} (partial clearances due to conjugate formation) with increasing DF dose was found. CL_{DS} remained constant at all dose levels studied, including multiple doses. CL (plasma clearance of DF) was significantly lower (-36%) in young women compared to young men, mainly due to reduced CL_{DPG}. Cl was higher in women taking oral contraceptives (+53%) or who smoked (+35%), mainly because of increased glucuronidation. In liver cirrhosis CL_u (unbound plasma clearance of DF) was significantly lower (-42%) due to reduced formation rates of DPG and DAG. CL_u was markedly reduced (-76%) in patients with renal failure (CL_{cr} = 18.4 $\pm$ 3.7 ml/min), possibly due to systemic deconjugation of DAG. Bile collection via T-tube after cholecystectomy showed increased biliary excretion of DPG and DAG in patients with impaired kidney function. Reduced elimination of DF in renal failure may therefore also involve enterohepatic cycling. Probenecid coadministration lead to a significant decrease in CL (-41%) due to impaired CL_{DPG} (-45%) and CL_{DAG} (-54%). CL_{DS} was not affected. Inhibition of DF glucuronidation by probenecid is probably the major cause. These studies indicate that, in case of DF, sulfation seems less readily perturbed than glucuronidation.

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DISPOSITION OF BHA AND BHT IN ANIMALS AND MAN

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At present there is *sufficient* respectively *limited* evidence that the synthetic food antioxidants BHA and BHT are carcinogenic in rodents. The main target tissues are the forestomach (BHA) and liver (BHT). BHA affects other gastrointestinal tract tissues as well. as indicated by an increase in tissue labelling index (which is considered an early marker of the tumorigenic potential of BHA). In general, BHA and BHT are negative in short-term tests for genotoxicity. Therefore a threshold for their carcinogenicity is assumed to exist and risk assessment for BHA and BHT is based on non-stochastical concepts, although the mechanism underlying their tumorigenicity is not known.

Studies on interspecies-comparison of metabolism and kinetics may contribute to a more sophisticated risk assessment. On administration of a single high dose to rats (200 mg/kg) and low dose to man (0.5 mg/kg), plasma BHA levels in either species were within one order of magnitude, unlike BHT, indicating an inter-species difference in the fate of BHA. In this experiment, recovery of BHA from urine and feces was 95 ± 10% (rat) and 49 ± 7% (man).

Upon continuous administration of BHA (2% of the diet) to rats during 2 weeks, metabolism of BHA to its O-demethylated metabolite *tert*-butylhydroquinone (TBHQ) was not found to be related to an increase in forestomach labelling index. On administration of BHA (0.5 mg/kg/day) to 8 volunteers during 10 days the amount of metabolites of BHA (i.e. conjugates of BHA and TBHQ) excreted into urine increased gradually; no other effects of BHA (clinical parameters in plasma and urine, modulation of phase-I and -II biotransformation capacity) were noticed in this study. However, recent data suggest that biotransformation of BHA is yet involved in the unknown mechanism underlying the carcinogenicity of BHA in rodent forestomach. Metabolism to quinones and subsequently to (hitherto unidentified) sulfur-containing compounds may give rise to formation of reactive oxygen species. In particular hydroxylradicals, may lead to the formation of DNA-adducts, misreading of DNA-templates, and thus form a genetic basis for cancer. The possible formation of these hypothesized metabolites of BHA in rodents and in man under the experimental conditions described above warrants further investigation. The key-question for risk assessment is whether or not this hypothesized mechanism is thresholded.

TRANSPORT OF PEPTIDE DRUGS ACROSS BIOLOGICAL MEMBRANES

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In general peptide drugs show high potency and low toxicity, making these compounds attractive as therapeutic agents. However, due to their unfavourable physicochemical properties peptides are very poorly transported across biological membranes. These include both the epithelial mucosa of the intestinal tract and other absorptive surfaces like the buccal, nasal and pulmonary epithelia. An approach to facilitate peptide transport is the use of protease inhibitors and particularly absorption enhancers, being capable of decreasing the enzymatic degradation and increasing the permeability of the epithelial tissues for peptides. A major problem encountered with the application of enhancers is their possible irritating and membrane damaging effects. Therefore, research in this field is directed towards the search for non-destructive and safe absorption enhancing compounds from endogenous origin.

This presentation will focus on the effect of a relatively new class of compounds, cyclodextrins, on nasal (poly)peptide absorption. Cyclodextrins are cyclic oligosaccharides of 6,7 or 8 glucose units (α-, β- and γ-cyclodextrins), which are able to form inclusion complexes with lipophilic drugs. Cyclodextrins may also exert direct effects on epithelial membranes, thereby facilitating transport of hydrophillic drugs like peptides. Comparative studies in rats with a variety of cyclodextrins showed that in particular dimethyl-β-cyclodextrin is a potent promoter of nasal insulin absorption, leading to almost complete insulin bioavailability. The influence of these formulations on the nasal ciliary movement, as measured in vitro, appeared to be moderate and reversible. Remarkable enhancing effects by cyclodextrins on nasal epithelial drug transport have also been found for other peptides. Thus cyclodextrins may have potential for efficient and safe nasal delivery of peptide drugs.

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MERCAPTURIC ACIDS AS A TOOL IN BIOTRANSFORMATION AND BIOMONITORING STUDIES

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Mercapturic acids are S-substituted N-acetyl-L-cysteine derivatives, which are metabolic end products of an important pathway in the biotransformation of electrophilic xenobiotics, notably the glutathione (GSH)-conjugation pathway. During the last few years the measurement of mercapturic acids in urine has become increasingly popular. On the one hand this is because their chemical structure reflects the mechanism of GSH-conjugation *in vivo*, and on the other hand, because their excretion in urine might reflect the exposure of a living organism to potentially toxic electrophilic compounds.

In this communication some recent examples illustrating the possibilities and limitations of using urinary mercapturic acids as a tool in biotransformation, toxicity and biomonitoring studies will be discussed. Thus mercapturic acid excretion has been shown to reflect the regio- and stereoselective GSH-conjugation of trichloroethylene and bromisoval. The latter compound has been suggested to be a non-invasive *in vivo* probe to assess isoenzyme-selective GSH-conjugation activity in experimental animals and perhaps in humans. Mercapturic acids are also useful precursor compounds in cases where GSH-conjugation, either directly (e.g. in the case of 1,2-dichloroethane) or indirectly (e.g. halogenated ethylenes) is leading to mutagenicity, carcinogenicity or to other organselective toxicities. Using N-trideutero-acetylated mercapturic acids the relevance of the balance of deacetylation/reacetylation in relation to the *in vivo* metabolism and (renal) toxicity of cysteine-conjugates and mercapturic acids has been determined.

Finally, the measurement of urinary mercapturic acids is becoming a more popular tool in the compound selective biomonitoring of exposure to electrophilic chemicals. Urinary 2-hydroxyethyl mercapturic acid thus may reflect exposure of animals and man to at least 5 different reactive intermediates originating from metabolism of a variety of electrophilic and therefore potentially toxic xenobiotic chemicals. Moreover, apart from internal dose assessment, the urinary excretion kinetics may reflect the toxicokinetics of electrophilic parent compounds. Both aspects will be illustrated with occupational and non-occupational exposure studies with Z- and E-1,3-dichloropropene, a frequently used nematocide.

THE DISPOSITION OF ICI 118630 IN ANIMALS AND MAN

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ICI 118,630 (ZOLADEX) is a an analogue of LH-RH, developed for use in a range of hormone dependant conditions. Extensive radiochemical support permitted the synthesis of the compound in two radioactive forms, which were administered to both laboratory animals and human volunteers. Clearance was fairly rapid, ($t_{½}$ 1.5-4h) in all species and proceeded via a combination of metabolism and excretion. Metabolite identification was established via HPLC and tandem MS. Some species variability was observed - in rat, rabbit and man, the major circulating component was 1-7, in dog it was parent compound and 5-10. In rat and mouse, excretion was via both urine and bile; urine was the major route of excretion in the dog and rabbit and urinary excretion accounted for all the dose in man. Some parent compound was detected in the urine of all species. The major urinary component was 5-10 (4-10 in dog) together with a range of further hydrolytic products. Only trace amounts of parent compound were detected in bile, where the major component was 5-7. There were few differences in the metabolite patterns of the two labelled forms. The primary site of metabolism appeared to be between residues 4 and 5 in all species except the dog.

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RENAL AND HEPATIC STRUCTURE-ACTIVITY STUDIES WITH RAT AND BOVINE C-S LYASE ENZYMES

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C-S lyase enzymes mediate a toxifying deviation of the normal glutathione conjugate metabolic pathways. In order to investigate the structure-activity relationships of C-S lyase enzymes from bovine and rat, the following conjugates were synthesized: dichlorovinyl- (DCVC), allyl-, 3-chloroallyl-, 2-chloroallyl-, 1-chloropropenyl-, phenyl-, 2-chlorophenyl-, 4-chlorophenyl-, 2,4-dichlorophenyl-, 3,4-dichlorophenyl-, 2,4,5-trichlorophenyl-, 4-bromophenyl-, and benzothiazolyl-L-cysteine. These compounds were incubated with partially purified C-S lyase enzyme preparations obtained from rat hepatic and renal tissues, and with corresponding preparations from bovine tissues. C-S lyase activity was determined by assaying the incubation mixtures for pyruvic acid.
DCVC and 1-chloropropenyl-L-cysteine (10mM) were the most efficient alkenyl substituted substrates in both rat liver and rat kidney preparations with specific activities (nmol/min/mg) of 2.89 and 1.28 respectively (rat liver) and 2.90 and 0.31 (rat kidney). Similar patterns were observed for the bovine enzymes; the specific activity for DCVC with bovine kidney was, however, greater at 9.13 nmol/min/mg. 2,4,5-Trichlorophenyl-L-cysteine (0.75mM) was the most effective aromatic substrate for rat liver and kidney C-S lyase (0.91 and 0.21). These results were confirmed in studies using the bovine enzymes. We suggest that a halogenated double bond cysteinyl thioether is important for activity in the alkenyl conjugates, and that the number of electronegative substituents is critical for the aromatic conjugates.
We acknowledge the financial and technical support of the Health and Safety Executive and ICI-CTL (studentship to AJW).

SEX DIFFERENCES IN DRUG METABOLISMS

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Over 15 different forms of cytochrome P-450 (P-450) are isolated from rat livers and some are shown to appear sex-specifically in either one of the sexes. These P-450s are mostly expressed liver specifically and regulated developmentally. All the sex-specific or sex-dominant P-450s investigated so far are shown to be under the influence of pituitary growth hormone, although the responses are not pleiotropic, and differ among each form. In addition, thyroid hormone has recently been shown to modulate the sex-specific expression of hepatic P-450s. In rat liver, sex-related difference is not limited on P-450s. Cytosolic sulfotransferases catalyzing sulfation of steroids (alcohols) and phenols appear in livers predominantly in adult female and male rats, respectively, and their hepatic expression is also regulated by pituitary growth hormone. The sex-related difference in drug metabolisms is not usually observed in man, and no clear evidence is available for the difference at isozyme levels. Data obtained from rats are often not applicable to predict the metabolic profiles in human. In these cases, The rat-specific phenomenon, sex-related difference, may need to take in consideration for the extrapolation. However, drug metabolizing activities in livers change with development in both rat and human, in which endocrine hormones are suggested to contribute. These results may imply the similarity of the mechanistic basis of age-related alteration of drug metabolizing activities to that of the sex-related difference in rats and mice.

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MODULATION OF XENOBIOTIC METABOLISM BY DIET AND NUTRITION

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Diet and nutrition are known to have important effects on the metabolism of drugs and other xenobiotics. This presentation deals with the mechanisms by which dietary factors modulate the levels of specific cytochrome P450 isozymes and the metabolism of xenobiotics as well as the biological consequences of these modulations. Diallyl sulfide (DAS) (a component of garlic oil) induces the level of hepatic P4502B1 in rats by transcriptional activation. A similar mechanism may be involved in the induction of P450IA1 and P450IA2 by indole-3-carbinol (derived from cruciferous vegetables). The induction of these enzymes may alter carcinogen and estrogen metabolism and affect carcinogenesis. DAS inactivates P4502E1 *in vivo* due to suicide inhibition of this enzyme by diallyl sulfone, a metabolite of DAS. Phenethyl isothiocyanate (PEITC) (derived from cruciferous vegetables) inactivates P4502E1 similarly. DAS and PEITC also competitively inhibit P4502E1-catalyzed reactions. *In vivo*, these compounds inhibit the metabolism, reduce the toxicity, and alter the carcinogenicity of many environmental chemicals. These dietary chemicals also affect the metabolism of xenobiotics catalyzed by other P450s. For example, PEITC is an effective inhibitor of NNK metabolism and carcinogenesis in mouse lung. Fasting and high fat diet induce the hepatic levels of P4502E1 and P4503A. Ketosis may be a factor involved in the induction of P4502E1 which is also induced by acetone. An elevated mRNA level was observed in the induction of P4502E1 by fasting, but not in the induction by acetone. (Supported by grants ES03938 and CA46535 from NIH and a grant from AICR).

MICROBIAL METABOLISM OF XENOBIOTICS IN THE ENVIRONMENT

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Microorganisms are in general responsible for the elimination, that means the mineralization, of xenobiotics in the environment. The extent of mineralization and the formation of possible stable intermediates depends on three factors, namely (i) the chemical structure of the compound, (ii) the environmental parameters, and (iii) the composition of the microbial population present. Environmental parameters comprise physical elements like temperature, accessibility of a compound, etc. and chemical elements like pH, redox, concentrations, etc. The composition of the microbial population is the result of the composition and the character of the xenobiotics, the environmental conditions, and the genetic information present in a given environmental compartment.

The three factors controlling the microbial transformation of xenobiotics will be discussed using chlorinated solvents and hexachlorocyclohexane as examples. Particular emphasis will also be given to molecular processes which form the base for adaptation and transformations in absence of molecular oxygen.

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ABSTRACTS

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DUAL EFFECT OF TRIAZOLODIAZEPINE DERIVATIVE, E6123 ON MONOOXYGENASE OF DOGS

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Triazolodiazepine derivative, E6123, (S)-(+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8H-pyrido-[4',3':4,5] thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine was a very potent inhibitor of PAF-induced response in vivo [IC_{50};1μg/kg p.o. (PAF-induced bronchoconstriction in guinea pigs)] . We have reported that the pharmacokinetics of E6123 were far better than those of some PAF-antagonists. The present study investigates the inhibitory and inductive effect of E6123 on liver microsomal monooxygenase of dogs. At 0.2 mg/kg daily dose for 3 months, there was slight inductive effect of E6123. At higher dose (1 and 5 mg/kg), E6123 decreased the total P-450 contents, however, the level of P-450 IIB increased and there was no change in that of P-450 IIIA in dogs. There was no significant decrease in N-demethylase activities measured toward benzphetamine and aminopyrine. N-demethylase activities of both benzphetamine and aminopyrine also did not increase,though the contents of P-450 IIB increased. E-6123 was found to prolong antipyrine half life, and increase AUC of antipyrine in dose dependent manner after single oral dose (1 and 10 mg/kg). This is seemed for monooxygenase activities to be compensated by dual effect of E6123.

There was no significant change in metabolic activity of E6123 with microsomal preparation induced both P-450 IIB and IIIA, as comparing with untreated microsomes in dogs. In conclusion, E6123 appeared as a P-450 IIB inducting drug without catalyzing metabolic activities of E6123, itself.

LACK OF STEREOSELECTIVITY OF PEROXISOME PROLIFERATION BY 2-PHENYLPROPIONIC ACID (2-PPA) INDICATES THAT ITS ACYL CoA DOES NOT PLAY A ROLE IN THIS PHENOMENON

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The significance of disturbances of lipid metabolism caused by xenobiotic acyl CoAs as possible causes of peroxisomal proliferation has been studied with the enantiomers of 2-PPA, the R-enantiomer of which is converted to the acyl CoA in rats while its S-antipode is not (1). *rac*-2-PPA (250 mg/kg/day i.p. x 3) was shown to be an hepatic peroxisomal proliferator in male SD rats on the basis of increases in microsomal cytochrome P450 content and lauric acid hydroxylation and hepatic CN^--insensitive palmitoyl CoA oxidase, a peroxisomal marker enzyme, while electron microscopy revealed a rise in the peroxisome/mitochondria ratio in hepatocytes. Further studies established the dose-response relationships for these changes. The R- and S-enantiomers were administered at a dose of 50 mg/kg/day i.p. x 3 and both were peroxisome proliferators of very similar potency. The effects of 100 mg/kg/day i.p. x 3 of the racemate, a dose giving ca. 75% of maximal response, were essentially additive of those of 50 mg/kg/day i.p. x 3 of its two component isomers. The stereoselectivity of acyl CoA formation from the enantiomers of 2-PPA was confirmed by their differential inhibition of microsomal palmitoyl CoA synthesis. Taken together, these data indicate that it is very unlikely that the acyl CoA of 2-PPA plays any rôle in the peroxisomal proliferation which this compound causes.

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DISPOSITION OF INTRAVENOUS HALOFANTRINE (HAL) AND ITS DESBUTYL METABOLITE (DES) IN BEAGLE DOGS

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HAL, a phenanthrene methanol antimalarial agent, has in vitro and in vivo activities against both chloroquine-sensitive and chloroquine-resistant Plasmodium falciparum. The major metabolite, DES, is equipotent with the parent compound against P. falciparum in vitro. The disposition kinetics of IV HAL was studied in 8 beagle dogs. HAL, 5 mg/kg, was administered by infusion pump over 10 min. Blood and plasma samples were collected at intervals over 35 days. Quantitation of HAL and DES in blood and plasma was by HPLC using fluorescence detection. The disposition of HAL followed a tri-exponential decline. The mean ($\pm$ SD) body clearance (CLb) from blood and plasma are 4.4 $\pm$ 1.7 l/h and 3.4 $\pm$ 1.1 l/h, respectively; while the elimination clearance (CLe) is 15.7 $\pm$ 4.3 l/h for blood and 12.9 $\pm$ 2.6 l/h for plasma. The distribution clearance (CLd) for blood is 11.3 $\pm$ 3.5 l/h and 9.5 $\pm$ 2.4 l/h for plasma. The steady-state volume of distribution (Vss) is 275.7 $\pm$ 165.8 l for blood, and 402.0 $\pm$ 205.6 l for plasma. The terminal half-lives (T 1/2) in blood and plasma are 111.3 $\pm$ 72.1 h and 192.8 $\pm$ 104.5 h, respectively. The mean residence time of HAL in the body (MRTb), central kinetic space (MRTc), and peripheral tissue space (MRTp) for blood are 79.6 $\pm$ 64.8 h, 0.27 $\pm$ 0.1 h, and 79.4 $\pm$ 64.8 h, respectively. The values for these parameters in plasma are 135.4 $\pm$ 85.6 h, 0.36 $\pm$ 0.2 h, and 135.0 $\pm$ 85.5 h, respectively. The fraction of the drug which enters the peripheral kinetic space for both blood and plasma is 0.7 $\pm$ 0.1. HAL is a low extraction drug with hepatic extraction ratios of 0.25 $\pm$ 0.09 for blood and 0.2 $\pm$ 0.06 for plasma. There is evidence of concentration of DES in RBCs.

KINETIC STUDIES ON RAT LIVER MICROSOMAL GLUTATHIONE TRANSFERASE

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The kinetic mechanism of both unactivated and activated (the enzyme is activated up to 15-fold by the sulfhydryl reagent N-ethylmaleimide) microsomal glutathione transferase was investigated using alternate substrate diagnosis. Non-paralell lines in Lineweaver-Burke plots were observed when the concentration of glutathione was varied against the identity of three electrophilic second substrates and when the concentration of 1-chloro-2,4-dinitrobenzene was varied against glutathione and two glutathione analogues. This pattern defines a random sequential mechanism for rat liver microsomal glutathione transferase. The microsomal glutathione transferase forms a Meisenheimer complex between 1,3,5-trinitrobenzene and glutathione. The formation constants were equal for the unactivated and activated enzyme ($15\pm1 \times 10^3$ and $14\pm1 \times 10^3$ M^{-1} respectively). Activation apparently does not alter the ability of the enzyme to stabilize this complex. The pH profile of K_{cat} (saturating 1-chloro-2,4-dinitrobenzene variable GSH) showed apparent pKa's of 6.4 and 6.8 for the unactivated and activated enzyme respectively, indicative of a rate determining deprotonation in the enzyme-GSH-1-chloro-2,4-dinitrobenzene complex. The rate behaviour of unactivated and activated rat liver microsomal glutathione transferase activity on glutathione concentration was examined utilizing a series of electrophilic halo-arene substrates. It was found that the K_{cat}/K_m values for glutathione increased greatly with the more electrophilic (reactive) substrates (1-chloro-2,4-dinitrobenzene and 4-chloro-3-nitrobenzacetophenone) upon activation (7.5- and 9-fold respectively). The efficiency for utilizing the less reactive substrates (4-chloro-3-nitrobenzamide, 2,5-dichloronitrobenzene and 1,2-dichloro-4-nitrobenzene) was also enhanced as evidenced by increases in the specific activity at low glutathione concentration (1.3-, 1.8- and 2.9-fold respectively). The potential for activation of microsomal glutathione transferase with the whole spectrum of varyingly reactive substrates can be considered as a well suited defence mechanism against toxic insult by electrophiles where glutathione levels are usually diminished.

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